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L10: Entry 23 of 37 File: USPT

DOCUMENT-IDENTIFIER: US 5997863 A
TITLE: Attenuation of wound healing processes

Abstract Text (1):
Glycosaminoglycans, including heparinases 1, 2 and 3 as well as chondroitinases AC and B from the Gram negative bacteria Flavobacterium heparinum, can be used either separately or in combination to manipulate cell proliferation. In one embodiment, heparinases are administered to degrade heparan sulfate components of the extracellular matrix, thereby allowing the heparin binding growth factors which are stored in the extracellular matrix to migrate to adjacent cells. The mobility of chemoattractant agents, growth factors and cells also can be increased by treating tissues with glycosaminoglycan degrading enzymes, both chondroitinases and heparinases. The enzymatic removal of chondroitin sulfates from cell surfaces effectively increases the availability of growth factor receptors on the cell's surface. Selectively removing heparan sulfate from cell surfaces while leaving the extracellular matrix intact, conversely, inhibits cell proliferation by down regulating the cell's response to growth factors. This is achieved by targeting heparin or heparan sulfate degrading activities to the cell surface. Targeting the heparin degrading activity can be achieved by genetically engineering a ligand binding functionality into the heparinase proteins, or by physically controlling the localized enzyme concentration through the method of administration.

Brief Summary Text (2):
The present invention describes a methodology for the use of glycosaminoglycan degrading enzymes to modulate events in the wound healing process.

Brief Summary Text (4):
For most cell types, events of mitogenesis and differentiation are subdued in the normal adult animal. These growth factor mediated events are more commonly associated with developing organisms, during wound healing processes or in various disease states including cancer and vascular disease. For example, the normal turnover rate of endothelial cells, including the lining of microvessels and arteries, is measured in thousands of days. During normal wound healing however, these endothelial cells proliferate rapidly, with a turnover rate of approximately five days (Folkman and Shing, J. Biol. Chem. 267(16):10931-10934, 1992). The increase in proliferation that occurs during wound healing appears to be the result of an increase in the local concentration of various angiogenic molecules, including growth factors.

Brief Summary Text (5):
The fibroblast growth factor family includes at least seven polypeptides that have been shown to stimulate proliferation in various cell lines including endothelial cells, fibroblasts, smooth muscle cells and epidermal cells. Included in this group are acidic fibroblast growth factor (FGF-1), basic fibroblast growth factor (FGF-2), int-2 (FGF-3), Kaposi sarcoma growth factor (FGF-4), hst-1 (FGF-5), hst-2 (FGF-6) and keratinocyte growth factor; (FGF-7) (Baird and Klagsbrun, Ann. N.Y. Acad. Sci. 638: xiv, 1991). These molecules, and other cytokines including tissue growth factors, TGF.alpha. and TGF.beta., platelet derived growth factors, PDGF, granulocyte-macrophage colony stimulating factor, GM-CSF, interleukin 3, IL-3, and platelet factor 4, PF4, share a common feature in their affinity for heparin (Clark, Dermatol. Clin. 11:647-666, 1993). Specific cell type responses also have been associated with particular factors. EGF and TGF.alpha. stimulate the proliferation of keratinocytes, TGF.beta. stimulates collagen and fibronectin synthesis, PDGF stimulates angiogenesis and granulation tissue formation and FGF-7 stimulates

epithelial cell proliferation (Staiano-Coico, et al., J. Exp. Med. 178:865-878, 1993). PDGF, FGF-2 and a recently described heparin binding epidermal growth factor HB-EGF (Higashiyama, et. al., Science 251:936-939, 1991) additionally are involved in the proliferation and migration of vascular smooth muscle cells and vascular endothelial cells.

Brief Summary Text (7):
An initial event in tissue or vessel injury may involve a mechanical dislodging of growth factors from the extracellular space, making them available to cell surface receptors where they stimulate cell proliferation and cell synthesis of additional growth factors. Alternately, cells under stress may secrete molecules which displace the extracellular growth factors from these storage reservoirs. Tumor cells have been shown to secrete degradative enzymes, including proteoglycanases, collagenases and metalloproteinases, coincident with metastasis (Nicolson Curr. Opinion Cell Biol. 1:1009-1019, 1989). In addition to facilitating tumor migration through blood vessels, the destruction of extracellular matrix components releases growth factors, thereby promoting new blood vessel formation which feeds the growing tumor mass (Folkman, et al., Am J Pathol 130:393-400, 1988).

Brief Summary Text (9):
The stimulation of cell proliferation and migration by growth factors constitutes one of the events in the wound healing process which is a multifactoral interactive process involving biochemical mediators, the extracellular matrix and parenchymal cells. The wound healing process is generally divided into three temporally overlapping phases: inflammation, proliferation and remodeling. During inflammation, blood borne cells infiltrate the wound site and release several mediating molecules including platelet derived growth factor, von Willibrand factor, thrombospondin, fibronectin, fibrinogen, 5-hydroxytryptophan, thromboxane-A2 and adenosine diphosphate (Kirsner and Eaglstein, J. Dermatol. 151:629-640, 1993). A platelet plug and thrombus are formed and provide a matrix for monocytes, fibroblasts and keratinocytes. Chemotactic molecules attract monocytes which transform into macrophages and secrete additional growth factors (Nathan and Sporn, J. Cell Biol. 113:981-986, 1991). Neutrophils may assist in this process by secreting the degradative enzymes elastase and collagenase which enhance the passage of cells through the basement membranes.

Brief Summary Text (10):
Keratinocytes and epidermal cells, which are involved in the closure of dermal wounds, migrate to the wound site during the proliferative phase. Angiogenesis, the formation of new blood vessels in response to chemoattractant and angiogenic signals (Folkman and Klagsbrun, Science 235:442-447, 1987), and fibroplasia, the accumulation of fibroblasts and formation of granulation tissue, also occurs during the proliferative phase. Tissue remodeling is accompanied by the secretion of matrix components, including fibronectin, collagen and proteoglycans which serve as a scaffold for cellular migration and tissue support. Type III collagen, synthesized in the earlier stages of wound healing, is replaced by the more permanent type I form through a process of proteolytic turnover.

Brief Summary Text (11):
Ischemia refers to the pathological condition due to the localized dysfunction of the vascular system resulting in inadequate blood supply with subsequent tissue damage. In this case revascularization, whether through the stimulation of angiogenesis or by surgical methods, must precede the normal wound healing course of the damaged tissue.

Brief Summary Text (12):
The action of enzymes which degrade components of the extracellular matrix and basement membranes may facilitate the events of tissue repair by a variety of mechanisms including the release of bound cytokines entrapped by heparan sulfate and by increasing the permeability of the matrix, thereby enhancing the mobility of mediator molecules, growth factors and chemotactic agents, as well as the cells involved in the healing process. Glycosaminoglycans are subject to degradation by a variety of eukaryotic and prokaryotic enzymes. Heparan sulfate degrading activity has been detected in platelets (oldberg et al. Biochemistry, 19:5755-5762, 1980), tumor cells (Nakajima, et al. J. Biol. Chem. 259:2283-2290, 1984) and endothelial cells (Gaal et al. Biochem. Biophys. Res. Comm., 161:604-614, 1989). These heparanase enzymes act by catalyzing the hydrolysis of the carbohydrate backbone of heparan

sulfate at the hexuronic acid (1.fwdarw.4) glucosamine linkage (Nakajima et al., J. Cell, Biochem., 36:157-167, 1988). Mammalian heparanases are typically inhibited by the highly sulfated heparin form of the heparin-heparan sulfate family. However, accurate biochemical characterizations of these enzymes have thus far been prevented by the lack of a method to obtain homogeneous preparations of the molecules.

Brief Summary Text (13):
Heparin degrading enzymes also have been found in microorganisms including Flavobacterium heparinum (Lohse and Linhardt, J. Biol. Chem. 267:2437-24355, 1992), Bacteroides strains (Saylers, et al., Appl. Environ. Microbiol. 33:319-322, 1977; Nakamura, et al., J. Clin. Microbiol. 26:1070-1071, 1988), Flavobacterium Hp206 (Yoshida, et al., 10th Annual Symposium of Glycoconjugates, Jerusalem 1989) and Cytophagia species (Bohn, et al., Drug Res. 41(I), Nr. 4:456-460, 1991). Chrondoitin sulfate degrading enzymes have been isolated from several microorganisms including Flavobacterium heparinum (Michaleacci, et al., Biochem. J. 151:123, 1975), Bacteroides species (Saylers, et al. J. Bacteriol. 143:781, 1980; Linn, et al., J. Bacteriol. 156:859, 1983; Steffen, et al., J. Clin. Microbiol. 14:153, 1981), Proteus vulgaris (Uamagata, et al., J. Biol. Chem. 243:1523, 1968, Suzuki, Meth. Enzymol. 28:911, 1972), Beneckea, Micrococcus and Vibrio species (Kitamikada and Lee, Appl. Microbiol. 29:414, 1975) and Arthrobacter aurescens (Hiyam and Okada, J. Biol. Chem. 250:1824-1828, 1975).

Brief Summary Text (14):
F. heparinum produces three forms of heparinase, heparinase 1, heparinase 2, and heparinase 3 (heparitinase) (Lohse and Linhardt, J. Biol. Chem. 267:24347-24355, 1992). All three enzymes cleave at glucosamine (1.fwdarw.4) hexuronic acid linkages with differing degrees of specificity depending on sulfation patterns and particular hexuronic acid residue, iduronic or glucuronic, in a particular cleavage site (Desai, et al., Arch. Biochem. Biophys. 306:461-468, 1993). F. heparinum also produces two enzymes which degrade members of the chondroitin sulfate/dermatan sulfate family. These are chondroitin lyase AC, which degrades both chondroitin sulfate A and chondroitin sulfate C by cleaving the galactosamine (1.fwdarw.4) glucuronic acid linkage in the polysaccharide backbone and chondroitin lyase B which degrades dermatan sulfate (chondroitin sulfate B) by cleaving the galactosamine (1.fwdarw.4) iduronic acid linkage in the polysaccharide backbone. The enzymatic mechanism of the F. heparinum enzymes is through an elimination reaction, thereby differentiating them from the mammalian glycosaminoglycan degrading enzymes. Furthermore, none of the F. heparinum lyase enzymes appear inhibited by glycosaminoglycan molecules as are the mammalian enzymes.

Brief Summary Text (15):
Mammalian heparanase, partially purified from tumor cell line extracts, as well as heparinase 1 and heparinase 3 from Flavobacterium heparinum, have been shown to release .sup.125 I radiolabelled FGF-2 that had been pre-adsorbed to extracellular matrix synthesized in vitro by bovine aorta endothelial cells (Bashkin, et al. J. Cell. Physiol. 167:126-137, 1992). However, since unfractionated and low molecular weight heparin elicited a similar release of the exogenously absorbed .sup.125 I radiolabelled FGF-2, it is not clear from these reports whether the measured release was due to the enzymatic degradation of the heparan sulfate in the ECM or an ion exchange type electrolytic displacement of FGF-2 from the negatively charged heparan sulfate. The same research group reported the release of growth promoting activity from vascular smooth muscle cells by treatment with heparinase 3 and from extracellular matrix by exposure to extracts of neutrophils or lymphoma cells. However, there has been no demonstration of the release of growth promoting activity from extracellular matrix by contact with bacterial glycosaminoglycan degrading enzymes nor have these enzymes been shown to promote tissue repair or new vessel growth in vivo.

Brief Summary Text (17):
It is a further object of the present invention to provide highly purified glycosaminoglycan degrading enzyme pharmaceutical compositions for use in enhancement of tissue repair and manipulation of angiogenesis.

Brief Summary Text (19):
Glycosaminoglycans, including heparinases 1, 2 and 3 as well as chondroitinases AC and

B from the Gram negative bacterium Flavobacterium heparinum, can be used either separately or in combination to manipulate cell proliferation. In one embodiment, heparinases are administered to degrade heparan sulfate components of the extracellular matrix, thereby allowing the heparin binding growth factors which are stored in the extracellular matrix to migrate to adjacent cells. The mobility of chemoattractant agents, growth factors and cells can also be increased by treating tissues with glycosaminoglycan degrading enzymes, both chondroitinases and heparinases. The enzymatic removal of chondroitin sulfates from cell surfaces effectively increases the availability of growth factor receptors on the cell's surface. Selectively removing heparan sulfate from cell surfaces while leaving the extracellular matrix intact, conversely, inhibits cell proliferation by down regulating the cell's response to growth factors. This is achieved by targeting heparin or heparan sulfate degrading activities to the cell surface. Targeting the heparin degrading activity can be achieved by genetically engineering a ligand binding functionality into the heparinase proteins, or by physically controlling the localized enzyme concentration through the method of administration.

Brief Summary Text (20):
Methods for preparing glycosaminoglycan enzymes and genetically engineered derivatives of them as well as methods for producing pharmaceutical preparations of highly purified glycosaminoglycan degrading enzymes are described. Methods are disclosed to produce derivatives of the heparin degrading enzymes which incorporate binding properties of other proteins. These molecules can be used to target the heparin degrading activity to the cell surface which inhibit a cell's response to endogenous growth factors.

Drawing Description Text (2):
FIGS. 1a, 1b, and 1c are schematic diagrams depicting the function of glycosaminoglycans in the extracellular matrix (ECM--top half) and on cell surfaces (bottom half). FIG. 1a shows that the heparan sulfate component (plain squiggled line) of heparan sulfate proteoglycans (HSPG) binds to heparin binding growth factors (HBGF) in both the extracellular matrix and at the cell surface. Growth factors not bound to heparan sulfate are unable to bind their cell surface receptor. Heparan sulfate or fragments of heparan sulfate attach to the growth factors and elicit a conformational change which allows binding to the receptor. Chondroitin sulfate (hatched squiggled line) proteoglycans (CSPG) also are located in the extracellular matrix and on the cell surface. At the cell surface the chondroitin sulfate molecules may sterically hinder the access of heparin binding growth factor receptors. FIG. 1b shows that treatment with chondroitin sulfate degrading enzymes allows greater access to the cell surface receptors and increases the mobility of molecules such as chemoattractants, growth factors and cells through the extracellular matrix. FIG. 1c shows that treatment with heparin or heparan sulfate degrading enzymes releases heparan sulfate fragments and heparin binding growth factors from the extracellular matrix, thereby increasing their availability to the adjacent cell surface receptors, and increases the mobility of molecules such as chemoattractants, growth factors and cells through the extracellular matrix.

Drawing Description Text (3):
FIG. 2 is a graph of the desorption (penetration into agarose (mm) over time (minutes)) of heparinase into semi-solid gels to measure the amount of enzyme present.

Drawing Description Text (4):
FIG. 3 is a graph of relative growth promoting activity released from enzyme treated extracellular matrix (x control) for untreated, heparinase 1, heparinase 2, heparinase 3, chondroitinase AC and chondroitinase B. The results are expressed as the ratio of thymidine incorporation by Balb/c 3T3 fibroblasts exposed to enzyme treated matrix supernatants to that of untreated matrix supernatants.

Drawing Description Text (5):
FIG. 4 is a graph of the relative growth promoting activity released from enzyme treated bovine corneas (x control) for untreated, heparinase 1, heparinase 2, and heparinase 3. The results are expressed as the ratio of thymidine incorporation by Balb/c 3T3 fibroblasts exposed to enzyme treated corneal supernatants to that of untreated corneal supernatants.

Drawing Description Text (6):
FIG. 5 is a graph of the release of .sup.35 S from extracellular matrix (cpm) for untreated, heparinase 1, heparinase 2, heparinase 3, chondroitinase AC, and chondroitinase B.

Drawing Description Text (7):
FIG. 6 is a graph of the relative adsorption of FGF-2 by enzyme treated balb/C 3T3 fibroblasts (% of control) for untreated, heparinase 1, heparinase 2, and chondroitinase AC.

Detailed Description Text (2):
A methodology for controlling events involved in wound healing processes by the use of highly purified glycosaminoglycan degrading enzymes derived from Flavobacterium heparinum genes is disclosed. Glycosaminoglycans, including heparan sulfate, chondroitin sulfate and dermatan sulfate, are the sulfated polysaccharide components of proteoglycans located on cell surfaces, where they act as cytokine receptors and in the extracellular space where they form the structure of the extracellular matrix and serve as a storage reservoir for growth factors. Glycosaminoglycan degrading enzymes from F. heparinum: heparinase 1 (EC 4.2.2.7), heparinase 2, heparinase 3 (EC 4.2.2.8), chondroitinase AC (EC 4.2.2.5) and chondroitinase B modulate the interactions involved in cell proliferation and migration by i) releasing heparin binding growth factors and molecules from the extracellular matrix, thereby increasing their availability to adjacent cells for the stimulation of proliferation and migration, ii) degrading components of the extracellular matrix, thereby facilitating the mobility of cytokines, chemoattractants and cells, iii) removing chondroitin sulfate from cell surfaces, thereby increasing access to cell surface receptors and iv) inhibiting the proliferative response of cells to growth factors by removing the heparan sulfate component of their growth factor receptor complex.

Detailed Description Text (3):
Heparin binding growth factor-receptor interactions require the presence of a third component: heparan sulfate, which is present on cell surfaces, or can be added to the cells, or released lytically as a heparan sulfate fragment from the extracellular matrix. The addition of heparin or heparan sulfate degrading enzymes in the range of between 0.001 and 5 IU/ml promotes cell proliferation by co-releasing heparin binding growth factors and heparan sulfate fragments from the extracellular matrix and increasing their availability to adjacent cells.

Detailed Description Text (4):
Selectively removing heparan sulfate from cell surfaces while leaving the extracellular matrix intact, conversely, inhibits cell proliferation by down regulating the cell's response to growth factors. This is achieved by targeting heparin or heparan sulfate degrading activities to the cell surface. Targeting the heparin degrading activity can be achieved by genetically engineering a ligand binding functionality into the heparinase proteins, or by physically controlling the localized enzyme concentration through the method of administration. For example, permeable double balloon catheters can direct heparinases, preferentially, to exposed vascular smooth muscle cells in injured vessels.

Detailed Description Text (5):
Preparation of Glycosaminoglycan Degrading Enzymes

Detailed Description Text (6):
D Glycosaminoglycan lysase enzymes can be prepared by isolation from bacterial or mammalian cells, either those which naturally produce the enzymes or have been genetically engineered to produce the enzymes.

Detailed Description Text (7):
Isolation of Naturally Produced Enzymes.

Detailed Description Text (8):
Glycosaminoglycan lyase enzymes can be purified from cultures of Flavobacterium heparinum, as follows. F. heparinum is cultured in 15 L computer controlled fermenters, in a variation of the defined nutrient medium described by Galliher et

al., Appl Environ. Microbiol. 41(2):360-365, 1981. For fermentations designed to produce heparin lyases, semi-purified heparin (Celsus Laboratories) is included in the media at a concentration of 1.0 g/L as the inducer of heparinase synthesis. For fermentations designed to produce chondroitin lyases, chondroitin sulfate A (Sigma) is included in the media at a concentration of 1.0 g/L as the inducer of chondroitinase AC and chondroitinase B synthesis. For both types of fermentation, the cells are harvested by centrifugation and the desired enzymes released from the periplasmic space by a variation of the osmotic shock procedure described by U.S. Pat. No. 5,169,772 to Zimmermann, et al. (1992).

Detailed Description Text (10):
The heparinase enzymes obtained by this method are greater than 98.5% pure as estimated by reverse phase HPLC analysis (BioCad, POROS II). Purification results for the heparinase enzymes are shown in Table 1.

Detailed Description Text (11):
Osmolates obtained from F. heparinum fermentations induced with chondroitin sulfate A are subjected to centrifugation to remove cells and cell debris and the supernatant applied to a cation exchange column (5.0 cm.times.30 cm, Sepharose.TM. S Big Beads, Pharmacia) at a linear flow rate of 10 cm.multidot.min.sup.-1. The bound proteins are eluted at a linear flow rate of 5.1 cm.multidot.min.sup.-1 with step gradients of 0.01 M phosphate, 0.01 M phosphate/0.25 M sodium chloride and 0.01 M phosphate/1.0 M. sodium chloride, all at pH, 7.0.+-.0.1. Chondroitinase activity elutes in the 0.25 M sodium chloride fraction which is further purified by diluting the chondroitinase containing fraction two-fold with 0.01 M sodium phosphate and applying the material onto a column containing cellufine sulfate (2.6 cm i.d..times.100 cm, Amicon) and eluting at a linear flow rate of 1.88 cm.multidot.min.sup.-1 with a linear gradient of sodium chloride, 0.0 to 0.4 M. Chondroitinase AC primarily elutes at 0.23 to 0.26 M sodium chloride while chondroitinase B eluted at 0.27 to 0.3 M sodium chloride. Each fraction was diluted two-fold with 0.01 M sodium phosphate and applied to a hydroxylapatite column (2.6 cm i.d..times.30 cm). The bound proteins are eluted with a step gradient of 0.25 M sodium chloride followed by a linear gradient of 0.25 to 1.0 M sodium chloride all in 0.025 M sodium phosphate at pH 7.0.+-.0.1. Chondroitinase B elutes in the 0.25 M sodium chloride step while chondroitinase AC elutes at 0.85 to 0.95 M sodium chloride. The chondroitinase B fraction is diluted two-fold in 0.01 M sodium phosphate and applied to a strong cation exchange column (CBX-S, J. T. Baker, 1.6 cm i.d..times.10 cm). The bound material is eluted at a flow rate of 1.0 cm.multidot.min.sup.-1 with a linear gradient from 0.125 to 0.325 M sodium chloride in 0.025 M sodium phosphate at pH 7.0.+-.0.1. Chondroitinase B elutes in a protein peak at 0.175 to 0.225 M sodium chloride and contains a minor contaminating protein of molecular weight 20,000 D. This protein is removed by gel filtration chromatography by loading the chondroitinase B sample onto a Superdex.TM. 200 column (1.0.times.30 cm, Pharmacia) and eluting with 0.05 M sodium phosphate, pH 7.2 at a linear flow rate of 1.25 cm.multidot.min.sup.-1 and collecting the protein containing fractions. The chondroitinase AC fraction collected from hydroxylapatite chromatography is diluted three-fold in 0.01 M sodium phosphate and applied to a strong cation exchange column (CBX-S, J. T. Baker, 1.6 cm i.d..times.10 cm). The bound material is eluted at a flow rate of 1.0 cm.multidot.min.sup.-1 with a linear gradient from 0.125 to 0.325 M sodium chloride in 0.025 M sodium phosphate at pH 7.0.+-.0.1. Chondroitinase AC elutes in a single protein peak at 0.175-0.225 M sodium chloride. Purification results for the chondroitinase enzymes are shown in Table 2.

Detailed Description Text (12):
Isolation of Recombinant Enzymes.

Detailed Description Text (13):
Glycosaminoglycan degrading enzymes also can be isolated from recombinant expression systems such as the heparinase 1 expression system described by Sasisekharan, et al., Proc. Natl. Acad. Sci. USA 90:8660-8664, 1993; the heparinase II and III expression systems disclosed in U.S. patent application Ser. No. 08/258,639 "Nucleic Acid Sequences and Expression Systems for Heparinase II and Heparinase III Derived From Flavobacterium heparinum" by Su, et al., filed Jun. 10, 1994; or the chondroitinase AC and B expression systems disclosed in U.S. patent application Ser. No. (B258000677US) "Chondroitin Lyase Enzymes" by Bennett, et al., filed Jul. 8, 1994, the teachings of which are incorporated herein. In these expression systems, the F. heparinum genes are

isolated and cloned into plasmids downstream from an inducable promoter. The plasmids are introduced into E. coli and the expression of the desired enzyme directed by a suitable induction method such as temperature shift and addition of IPTG to the medium.

Detailed Description Text (14):
The enzymes can be recovered in a purified form by a modification of the methods described herein. Cell disruption is achieved by homogenization, sonication or enzyatic treatment to break the cell wall and release cytoplasmic components. If enzyme synthesis results in aggregation, the aggregate can then be dissolved by a denaturing agent, 3 to 6 M guanidine HCl or 4 to 8 M urea and the protein refolded by removal of the denaturing agent through dialysis or dilution. The refolded enzyme can be further purified using the liquid chromatographic methods described above.

Detailed Description Text (16):
Fusion proteins incorporating glycosaminoglycan degrading enzymes ligated to proteins with specific binding properties can be created by recombinant molecular biology techniques. By choosing an appropriate binding protein, the glycosaminoglycan degrading activity can be targeted to specific sites in vivo. For example, epidermal growth factor binds cell receptors expressed preferentially on the surface of smooth muscle cells as described by Pickering, et al., J Clin Invest, 91:724-729, 1993. Fusion proteins containing this moiety ligated to a heparinase protein direct heparin or heparan sulfate degrading activity to the surface of smooth muscle cells, thereby diminishing their response to available cytokines. This type of fusion protein is of value in combating disease states that result from overgrowth of smooth muscle cells such as the vascular conditions of atherosclerosis and re-occlusion of vessels following percutaneous transluminal coronary angioplasty.

Detailed Description Text (21):
Other binding proteins can be antibodies or antibody fragments that recognize specific cell markers, hormones or other molecules that are bound by cell surface receptors. An example of a hormone bound by certain cell types is estrogen, which is bound to a greater degree by certain types of cancer cells. Another example is melanin, which is also present in higher concentrations of certain cancer cells. Antibodies to many specific cell surface markers are known.

Detailed Description Text (23):
Methods for extending the in vivo half-life are known and routinely used, especially in the case of enzymes. Examples of suitable methods using attachment of polyethylene glycol moieties to the protein, which inhibits uptake by the reticuloendothelial system. Preparation and characterization of "peglyated" proteins is described by Lu, et al., Pept. Res. 6(3), 140-146, 1993; Delgado, et al., Critical Rev. Ther. Drug Carrier Syst. 9(3-4), 249-304, 1992, the teachings of which are incorporated herein.

Detailed Description Text (25):
The enzymes can be administered topically, locally or systemically. Topical or local administration is preferred for greater control. The enzymes, alone or in combination, are mixed with an appropriate pharmaceutical carrier, then administered in an effective amount to produce the desired effect on the treated cells using methods known to those skilled in the art, for example, for topical application, by direct application to a site, or for local application, by means of injection or catheter.

Detailed Description Text (26):
Targeting and effective concentration dosages can be achieved by preparation of targeted enzymes as described above, or by the use of targeting vehicles, such as a catheter or polymeric delivery system, to achieve controlled site specific delivery of enzyme.

Detailed Description Text (28):
Glycosaminoglycan degrading enzymes can be mixed with a variety of common gels, creams or ointments to facilitate their application for treatment of dermal wounds. These gels or ointments can be administered alone or in a transdermal patch or bandage to facilitate penetration of an effective amount of enzyme to the cells which are to be treated.

Detailed Description Text (29):
Administration of Enzymes via Controlled Release Matrices or Injection:

Detailed Description Text (30):
Enzymes can also be formulated in a carrier for administration by injection, for example, in saline or an aqueous buffer, using standard methodology, or encapsulated in a polymeric matrix. Encapsulation of enzymes in controlled release formulations is well known; materials include but not limited to liposomes, lipospheres, biodegradable polymeric matrices, and vesicles. These encapsulants are typically microparticles having a diameter from 60 nm to 100 microns, but preferably less than ten microns, and more preferably one micron or less in diameter.

Detailed Description Text (31):
Proteosomes are prepared from outer membrane proteins of the Meningococcal bacteria and been reported to bind proteins containing hydrophobic anchors by Lowell, et al Science, 240:800 (1988). Proteosome proteins are highly hydrophobic, reflecting their role as transmembrane proteins and porins. When isolated, their hydrophobic protein-protein interactions cause them to form naturally multimolecular, membraneous 60 to 1000 nm vesicles or membrane vesicle fragments, depending on the strength of the detergent used in their isolation. The enzyme can also be encapsulated within a proteoliposome as described by Miller et al., J. Exp. Med. 176:1739-1744 (1992) and incorporated by reference herein, as described above with reference to proteosomes. Alternatively, the enzyme can be encapsulated in lipid vesicles such as Novasome.TM. lipid vesicles (Micro Vescular Systems, Inc., Nashua, N.H.). Another carrier is described in PCT US90/06590 by Nova Pharmaceuticals, the teachings of which are incorporated herein, which is referred to as a liposphere, having a solid core and an outer shell layer formed of phospholipid.

Detailed Description Text (32):
The carrier may also be a polymeric delayed release system. Biodegradable synthetic polymers are particularly useful to effect the controlled release of enzymes. Microencapsulation has been applied to the injection of microencapsulated pharmaceuticals to give a controlled release. A number of factors contribute to the selection of a particular polymer for microencapsulation. The reproducibility of polymer synthesis and the microencapsulation process, the cost of the microencapsulation materials and process, the toxicological profile, the requirements for variable release kinetics and the physicochemical compatibility of the polymer and the antigens are all factors that must be considered. Examples of useful polymers are polycarbonates, polyesters, polyurethanes, polyorthoesters and polyamides, particularly those that are biodegradable.

Detailed Description Text (34):
Enzymes can also be applied as films or implants, for example, to coat a tissue where growth is to be inhibited. Examples of materials used for controlled release which are administered as gels or films incorporating the agent to be released include Pluronics.TM. (BASF), copolymers of polyethylene oxide and polypropylene glycol.

Detailed Description Text (36):
The enzymes can be administered topically, as described above, or by injection. Typically, injection is performed using either a syringe or catheter. The advantage of the catheter is that material can be applied to surfaces such as the inside of blood vessels during a procedure such as angioplasty, where the goal is to inhibit restenosis by inhibiting the abnormal proliferation of cells that frequently follows the surgical procedure. Enzymes can also be administered simultaneously with surgery, so that healing of the wound is enhanced. Enzymes could also be admnistered during surgery to accelerate healing of the surgical wound. This could be accomplished by formulating the enzyme in a biocompatible gel or salve that would be applied directly to the wound site at the conclusion of the corrective procedure.

Detailed Description Text (37):
Glycosaminoglycan degrading enzymes can be applied intra-dermally to elicit an accelerated formation of new vessels in ischemic regions. Mechanistically, this is achieved by the dislodgment of growth factors from their extracellular storage reservoir where they are sequestered by heparan sulfate proteoglycans and by enhancing the mobility of cytokines and chemoattractants through the diseased tissue area.

Detailed Description Text (40):
Preparation of Topical Enzyme Compositions.

Detailed Description Text (43):
This experiment was repeated in the carboxymethyl cellulose/glycerol formulation using either 20 IU/ml chondroitinasese AC or 20 IU/ml chondroitinase B as the active ingredient and chondroitin sulfate A or dermatan sufate B as the test reagent. The results are shown in Table 3.

Detailed Description Text (45):
Preparation of a Heparinase or Chondroitinase Bandage.

Detailed Description Text (46):
The three bacterial heparinases and two chondroitinases, purified as described herein, were placed in solutions containing 0.01 M sodium phosphate, 0.2 M sodium chloride, pH 7.0 and 35 IU/ml enzyme. Semi-solid gels consisting of 4% polyethylene oxide (7.5 cm.times.5 cm.times.0.3 cm) were contacted with 6 ml enzyme solution for 3 h, during which time more than 70% of the enzyme solution absorbed into the gel matrix.

Detailed Description Text (47):
The enzyme containing gels were then tested for bioavailability (desorption) by the protamine precipitation of glycosaminoglycan--agarose gels as described herein. Enzyme containing patches were allowed to absorb to glycosaminoglycan-agarose gels for 90 minutes at 37.degree. C. before being transferred to a fresh agarose gel. The procedure was repeated for a total period of 7.5 hours. Semi-solid gels consisting of 4% polyethylene oxide (7.55.times.0.3 cm) were soaked in 6 to 8 ml heparinase 1 at a concentration of between 35 and 60 IU/ml for three hours during which time the enzyme was absorbed into the matrix. The matrices were applied to 1% agarose gels containing 0.05% heparin and incubated at 37.degree. C. Enzyme containing gels were transferred to fresh agarose gels each 90 minutes for a total of 7.5 hours. After incubation the agarose gels were contacted with 2.0% protamine sulfate to precipitate unfractionated glycosaminoglycan. Penetration of the enzymes was observed by measuring the depth of the clear zone in the precipitated agarose gels. The results are illustrated in FIG. 2.

Detailed Description Text (50):
Flavobacterial heparin degrading enzymes can dislodge substances exhibiting growth promoting activities from extracellular matrices. Primary endothelial cells were isolated from bovine corneal tissue and maintained in DMEM containing; 10% fetal calf serum, and 5% calf serum. Cells from confluent petri dishes were diluted 10-fold and grown in DMEM containing 10% fetal calf serum, 4% dextran and 5% calf serum, in 96-well plates for 12 to 14 days and were supplemented with FGF-2 at the rate of 0.5 ng/ml-day. The endothelial cells were removed by treatment with a solution containing 0.5% Triton and 0.02 M sodium hydroxide in phosphate buffered saline for 0.5 to 5 minutes, followed by three washes with phosphate buffered saline. This procedure yields plates coated with a layer of extracellular matrix which is stable for two years when stored at 4.degree. C. in phosphate buffered saline.

Detailed Description Text (51):
Varying amounts of the glycosaminoglycan degrading enzymes, purified as described herein, were added to the extracellular matrix in 0.2 ml/well containing 0.16% fetal calf serum--DMEM. Contacting with the glycosaminoglycan degrading enzymes was allowed to take place for 1 hour at 37.degree. C. The supernatants from these enzyme-extracellular matrix reaction mixtures were then tested for mitogenic activity by determining the incorporation of .sup.3 H-thymidine by quiescent balb/c 3T3 fibroblasts as described by Vlodavsky et al., Proc. Natl. Acad. Sci. 84:2292-2296, 1987.

Detailed Description Text (52):
Extracellular matrices formed in vitro from a primary endothelial cell line were treated with either heparinase 1, 2 or 3 at a concentration of 0.1 IU/ml, chondroitinase AC at a concentration of 1.0 IU/ml or chondroitinase B at a concentration of 0.5 IU/ml for 60 minutes. Reaction supernatants were tested for the presence of mitogenic activity by a thymidine incorporation assay. The results are

shown in FIG. 3.

Detailed Description Text (54):
Keparin and Heparan Sulfate Degrading Enzymes can also be Used to Release Growth Promoting Activity from Intact Animal Tissues.

Detailed Description Text (59):
Glycosaminoglycan degrading enzymes alter the extracellular matrix by cleaving the glycosaminoglycan components of the extracellular matrix proteoglycan. Preparation of extracellular matrix with .sup.35 S-sulfate containing proteoglycan and subsequent digestion of this radiolabelled matrix with Flavobacterial glycosaminoglycan degrading enzymes allows a quantitative assessment of the effect of the enzymes. .sup.35 S-sulfate containing extracellular matrix was produced by seeding dishes with primary bovine corneal endothelial cells grown to confluence in DMEM with 10% fetal calf serum and 5% calf serum diluted 10-fold into Fisher medium supplemented with 10% fetal calf serum, 5% calf serum, 4% dextran, and 25 .mu.Ci/ml Na.sub.2.sup.35 SO.sub.4 and cultured for 12 to 14 days with the addition of 0.5 ng/ml-day FGF-2. The endothelial cells were removed from the radiolabelled extracellular matrix by treatment with a solution containing 0.5% Triton, 0.02 M sodium hydroxide in phosphate buffered saline for 0.5 to 5 minutes, followed by three washes with phosphate buffered saline.

Detailed Description Text (60):
Extracellular matrix containing .sup.35 S sulfate in the glycosaminoglycan portion was treated with phosphate buffered saline or heparinases 1, 2 or 3, or chondroitinases AC or B at a concentration of 0.1 IU/ml in 1 ml/well dishes containing phosphate buffered saline, and the digestion was allowed to proceed for 1 hour at 37.degree. C. The amount of glycosaminoglycan released was determined by measuring the radiolabelled sulfate released to the supernatant with a Packard 1600 TR liquid scintillation counter. An estimate of 80,000 cpm was the total amount of radiolabelled sulfate contained in each reaction. The results are shown in FIG. 5.

Detailed Description Text (61):
The action of the Flavobacterial heparin degrading enzymes is extremely rapid, and the generation of .sup.35 S-sulfate labeled material occurs seconds after their addition to radiolabelled extracellular matrix as described above. In contrast, an equal amount of mammalian heparanase isolated from human placenta shows a 15 to 20 minute lag time after addition to the radiolabelled matrix before any measurable increase in the level of soluble .sup.35 S-sulfate labeled material is detected. This observation further differentiates the mammalian and bacterial enzymes.

Detailed Description Text (62):
While treatment of the extracellular matrix with glycosaminoglycan degrading enzymes alters the glycosaminoglycan component of the extracellular matrix proteoglycan, the overall structural integrity of the matrix remains unchanged as viewed by electron microscopy. Although structurally intact, enzymatically treated extracellular matrix exhibits enhanced permeability to macromolecules. This increased permeability can be demonstrated by examining the ability of the Flavobacterial glycosaminoglycan degrading enzymes to facilitate the passage of 25 nucleotide bases up to 2 Kb nucleotide fragments across a 0.45 micron pore polyethylene terephthalate (PET) membrane coated with extracellular matrix. Primary bovine corneal endothelial cells maintained as described above are diluted 1:10 from confluent dishes and seeded onto 0.45 micron pore PET membrane tissue culture inserts (Falcon) in DMEM supplemented with 10% fetal calf serum, 5% calf serum, 4% dextran, and cultured for 12 to 14 days with the addition of 0.5 ng/ml-day FGF-2. The endothelial cells are removed as described above, and the extracellular matrix coated PET inserts treated with either heparinase 1, 2, or 3 at a concentration 0.1 IU/ml, or with either chondroitinase AC or B at a concentration of 1 IU/ml in phosphate buffered saline at 37.degree. C. for 1 hour and rinsed three times with phosphate buffered saline.

Detailed Description Text (66):
Glycosaminoglycan degrading enzymes can attenuate a cell's response to growth factors by cleaving the glycosaminoglycan component of cell surface proteoglycans. Vascular smooth muscle cells were grown in 96 well plates in DMEM supplemented with 10% fetal serum until confluent. The cells were treated with either heparinase 1, 2 or 3 or chondroitinase AC at a concentration of 0.1 IU/ml for 1 hour at 37.degree. C., then

chilled on ice and washed twice with an incubation medium comprised of 0.025 M HEPES, 0.002 M Tris and 0.1% BSA in DMEM at pH 7.5. The cells were suspended in 0.25 ml incubation buffer containing 5 ng .sup.125 I-FGF-2 (0.5 .mu.Ci) and incubated at 4.degree. C. for 2 hours. Adsorption of FGF-2 to cell surface glycosaminoglycan was determined by washing the cells with an elution buffer consisting of 0.025 M HEPES and 2 M sodium chloride at pH 7.4, and measuring the recovered .sup.125 I with a gamma-counter (Wallac, Model 1740).

Detailed Description Text (67):
Balb/C 3T3 fibroblasts were treated with 0.1 IU/ml heparinases 1, 2 or 3, or chondroitinase AC and exposed to .sup.125 I-FGF-2. The amount of FGF-2 adsorbed to the cell surface glycosaminoglycan was determined by extracting the glycosaminoglycan bound fraction in 0.025 M HEPES, 2.0 M sodium chloride and measuring FGF-2 using a gamma counter and is expressed as a percentage of FGF-2 bound to untreated cells. The results are shown in FIG. 6.

Detailed Description Text (70):
Glycosaminoglycan degrading enzyme treatment of cell surfaces can either enhance growth factor binding as in the case of chondroitin degrading enzymes, or inhibit growth factor binding as in the case of heparin and heparan sulfate degrading enzymes. The removal of cell surface heparan sulfate can be compensated by heparin or heparan sulfate fragments released from the extracellular matrix by enzymatic treatment.

Detailed Description Text (72):
.sup.3 H-thymidine was included in the incubation and proliferation determined as described by Vlodavsky et al. Proliferation of vascular smooth muscle cells was monitored by thymidine incorporation and is expressed as a ratio of cells exposed to enzyme released material to that of untreated matrices for a) untreated ECM, untreated cells, b) heparinase 2 treated ECM, untreated cells, and c) heparinase 2 treated ECM, treated cells. The results are shown in FIG. 7.

Detailed Description Text (78):
The data indicate the potential utility of compositions containing one or a combination of the Flavobacterium heparinum derived glycosaminoglycan degrading enzymes for accelerating tissue repair in humans.

Detailed Description Paragraph Table (1):
TABLE 1 _____ Purification of heparinase enzymes from Flavobacterium heparinum fermentations activity specific activity yield sample (IU) (IU/mg) (%) _____ fermentation heparin degrading 94,500 100 heparan sulfate 75,400 ND 100 degrading osmolate heparin 52,100 55 degrading heparan sulfate 42,000 56 degrading cation exchange heparin degrading 22,600 24 heparan sulfate 27,540 ND 37 degrading cellufine sulfate heparin degrading 19,200 20 heparan sulfate 9,328 12 degrading hydroxylapatite heparinase 1 16,300 115.3 17 heparinase 2 2,049 38.41 heparinase 3 5,150 744.46

Detailed Description Paragraph Table (2):
TABLE 2 _____ Purification of chondroitinase enzymes from Flavobacterium heparinum fermentations specific activity activity yield sample (IU) (IU/mg) (%) _____ fermentation chondroitinase AC 65,348 0.764 100 chondroitinase B 21,531 0.252 100 osmolate chondroitinase AC 39,468 1.44 60 chondroitinase B 15,251 0.588 71 cation exchange chondroitinase AC 27,935 9.58 43 chondroitinase B 13,801 4.731 64 cellufine suifate chondroitinase AC 18,160 22.6 28 chondroitinase B 6,274 21.2 29 hydroxylapatite chondroitinase AC 14,494 146.8 22 chondroitinase B 3,960 65.62 18 strong cation exchange chondroitinase AC 9,843 211.4 15 chondroitinase B 4,104 167.2. 18 gel filtration chondroitinase B 2,814 278.7 13

Detailed Description Paragraph Table (3):
TABLE 3 _____ Enzymatic activity and desorption of heparinase 1 from pharmaceutical gel formulations. pharmaceutical activity desorption (mm) carrier enzyme (IU/ml) 1 hour 4 hour _____ PBS heparinase 1 100 4 ND 4% polyethy- heparinase 1 6.9 3 ND lene oxide carbomer gel heparinase 1 5.5 1 ND glycerol/CM cellulose heparinase 1 12.2 3 7 PBS chondroitinase

AC 16.4 3 ND glycerol/CM cellulose chondroitinase AC 12.5 1 3 PBS chondroitinase B 4.7 3 ND glycerol/CM cellulose chondroitinase B 6.5 1 4

Other Reference Publication (1):
Silver, P. (1988) IBT 9302 (Heparinase III): a novel enzyme for the management of reperfusion injury-related vascular damage, restinosis and wound healing. Exp. Opin. Invest. Drugs 7(6):1003-1014.

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Bohn, et al., "Fragmentation of Heparin by Enzymes from Newly Isolated Microorganisms," Drug Res. 41(1), Nr. 4:456-460 (1991).

Other Reference Publication (13):
Folkman and Shing, "Angiogenesis," J. Biol. Chem. 267(16):10931-10934 (1992).

Other Reference Publication (17):
Hiyam and Okada, "Crystallization and Some Properties of Chondroitinase from Arthrobacter aurescens," J. Biol. Chem. 250:1824-1828 (1975).

Other Reference Publication (24):
Michaleacci, et al., "A Comparative Study Between a Chondroitinase B and a Chondroitinase AC from Flavobacterium heparinum," Biochem. J. 151:123 (1975).

Other Reference Publication (38):
Steffen, et al., "Hydrolytic Enzymes of Anaerobic Bacteria Isolated from Human Infections," J. Clin. Microbiol., 14:153 (1981).

Other Reference Publication (39):
Suzuki, "Chondroitinases from Proteus vulgaris and Flavobacterium heparinum," Meth. Enzymol. 28:911 (1972).

CLAIMS:

administering to the cells around the wound bacterial glycosaminoglycan degrading lyases selected from the group consisting of heparinase 2 from Flavobacterium heparinum, heparinase 3 from Flavohacterium heparinum, chondroitinase AC from Flavobacterium heparinum, and chondroitinase B from Flavobacterium heparinum, heparinase from Bacteroides strains, heparinase from Flavobacterium Hp206, heparinase from Cytophagia species, chondroitin sulfate degrading lyases from Bacteroides species, chondroitin sulfate degrading lyases from Proteus vulgaris, chondroitin sulfate degrading lyases from Microcossus, chondroitin sulfate degrading lyases from Vibrio species, chondroitin sulfate degrading lyases from Arthrobacter aurescens, and combinations thereof in an amount effective to enhance normal wound healing.

9. A pharmaceutical composition comprising a bacterial glycosaminoglycan degrading lyase selected from the group consisting of heparinase 2 from Flavobacterium heparinum, heparinase 3 from Flavobacterium heparinum, chondroitinase AC from Flavobacterium heparinum, and chondroitinase B from Flavobacterium heparinum, heparinase from Bacteroides strains, heparinase from Flavobacterium Hp206, heparinase from Cytophagia species, chondroitin sulfate degrading lyases from Bacteroides species, chondroitin sulfate degrading lyases from Proteus vulgaris, chondroitin sulfate degrading lyases from Microcossus, chondroitin sulfate degrading lyases from Vibrio species, chondroitin sulfate degrading lyases from Arthrobacter aurescens, and combinations thereof in combination with a pharmaceutically acceptable carrier for localized administration of an effective amount to enhance normal wound healing.

15. A pharmaceutical composition comprising a bacterial glycosaminoglycan degrading lyase selected from the group consisting of heparinase 2 from Flavobacterium heparinum, heparinase 3 from Flavobacterium heparinum, chondroitinase AC from Flavobacterium heparinum, and chondroitinase B from Flavobacterium heparinum, in combination with a pharmaceutically acceptable carrier for localized administration of an effective amount to enhance normal wound healing, wherein the lyase is expressed from a recombinant nucleotide sequence in an organism in which it does not naturally occur and the lyase is processed differently than in the organism in which it

naturally occurs.

17. A pharmaceutical composition comprising a bacterial glycosaminoglycan degrading lyase selected from the group consisting of heparinase 2 from Flavobacterium heparinum, heparinase 3 from Flavobacterium heparinum, chondroitinase AC from Flavobacterium heparinum, and chondroitinase B from Flavobacterium heparinum in combination with a pharmaceutically acceptable carrier for localized administration of an effective amount to enhance normal wound heating, wherein the glycosaminoglycan degrading lyase is a fusion protein.